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Biochimica et Biophysica Acta 1587 (2002) 240–248

BIOCHIMICA ET BIOPHYSICA ACTA

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Review

The genetic basis of fluconazole resistance development in *Candida albicans*

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Received 24 January 2002; accepted 24 January 2002

Abstract

Infections by the opportunistic fungal pathogen *Candida albicans* are widely treated with the antifungal agent fluconazole that inhibits the biosynthesis of ergosterol, the major sterol in the fungal plasma membrane. The emergence of fluconazole-resistant *C. albicans* strains is a significant problem after long-term treatment of recurrent oropharyngeal candidiasis (OPC) in acquired immunodeficiency syndrome (AIDS) patients. Resistance can be caused by alterations in sterol biosynthesis, by mutations in the drug target enzyme, sterol 14α-demethylase (14DM), which lower its affinity for fluconazole, by increased expression of the *ERG11* gene encoding 14DM, or by overexpression of genes coding for membrane transport proteins of the ABC transporter (*CDR1*/*CDR2*) or the major facilitator (*MDR1*) superfamilies. Different mechanisms are frequently combined to result in a stepwise development of fluconazole resistance over time. The *MDR1* gene is not or barely transcribed during growth in vitro in fluconazole-susceptible *C. albicans* strains, but overexpressed in many fluconazole-resistant clinical isolates, resulting in reduced intracellular fluconazole accumulation. The activation of the gene in resistant isolates is caused by mutations in as yet unknown trans-regulatory factors, and the resulting constitutive high level of *MDR1* expression causes resistance to other toxic compounds in addition to fluconazole. Disruption of both alleles of the *MDR1* gene in resistant *C. albicans* isolates abolishes their resistance to these drugs, providing genetic evidence that *MDR1* mediates multidrug resistance in *C. albicans*.

Keywords: *Candida albicans*; Drug resistance; Efflux pump; Fluconazole; Major facilitator

1. Introduction

The yeast *Candida albicans* is a harmless colonizer of mucosal surfaces in many healthy persons, but it can cause superficial as well as life-threatening systemic infections in immunocompromised patients. Especially people infected with the human immunodeficiency virus (HIV) and acquired immunodeficiency syndrome (AIDS) patients frequently suffer from oropharyngeal candidiasis (OPC). Few classes of antifungal drugs are available to treat *Candida* infections. The azoles affect the biosynthesis of ergosterol, the major sterol in the fungal plasma membrane, by inhibiting the enzyme sterol 14α-demethylase (14DM), a cytochrome P-450 enzyme. This key enzyme in the ergosterol biosynthesis pathway catalyzes the oxidative removal of the 14α-methyl group from lanosterol. Azoles bind to the heme in the active site of 14DM, competing with substrate binding [1]. The triazole fluconazole is the most widely used drug to treat *Candida* infections, due to its favourable bioavailability and safety profile. The clinical response to fluconazole in patients with OPC is usually good but because of incomplete eradication of the fungi due to the fungistatic rather than fungicidal effect of azoles, relapses occur frequently [2]. The prolonged and repeated treatment of OPC in AIDS patients has resulted in an increasing frequency of therapy failures caused by the emergence of fluconazole-resistant *C. albicans* strains [3].

2. The development of fluconazole resistance in *C. albicans*

Especially in AIDS patients receiving long-term fluconazole therapy, clinical resistance is usually correlated with

Abbreviations: ABC, ATP-binding cassette; AIDS, acquired immunodeficiency syndrome; ATP, adenosine triphosphate; 14DM, sterol 14α-demethylase; GFP, green fluorescent protein; HIV, human immunodeficiency virus; 4-NQO, 4-nitroquinoline-*N*-oxide; OPC, oropharyngeal candidiasis

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0925-4439/02/$ - see front matter
PII: S0925-4439(02)00087-X

the isolation of *C. albicans* strains displaying decreased in vitro susceptibility to fluconazole [3,4]. To determine whether fluconazole resistance is due to the aquisition of a new, resistant strain that replaces a previously infecting, susceptible strain, or if resistance has developed in a previously susceptible strain that colonized the patient before, it is necessary to discriminate genetically unrelated *C. albicans* strains from each other. Many molecular typing methods are available for *C. albicans*, but Southern hybridization of genomic DNA with moderately repetitive DNA elements has been shown to have the highest reproducibility and discriminatory power (see Fig. 1). Molecular typing has demonstrated that colonizing populations of *C. albicans* are usually clonal, i.e. a single strain is in most cases the cause of recurrent infections in a patient, but the strain may evolve into subtypes [5]. However, it has also been shown that a patient can be colonized by several different *C. albicans* strains at the same time [6–8]. In many studies, only a single isolate is characterized from each infection episode. Therefore, a strain that was present as a minor constituent of the colonizing population in the patient may be isolated for the first time after it has acquired resistance and overgrown a previously dominating other strain, thereby giving the appearance of resistance being due to strain replacement. Although transmission of a resistant strain, for example from the sexual partner, may occur [9], the analysis of many *C. albicans* isolates from patients with recurrent infections has demonstrated that fluconazole resistance usually developed in a previously susceptible strain from the same patient [10,11]. Such series of matched isolates, i.e. isolates representing the same *C. albicans* strain but differing in their susceptibility to fluconazole, are an excellent tool to study the mechanisms of drug resistance, since genetic alterations in a resistant isolate as compared to a matched susceptible isolate are likely to be related to the resistant phenotype [4].

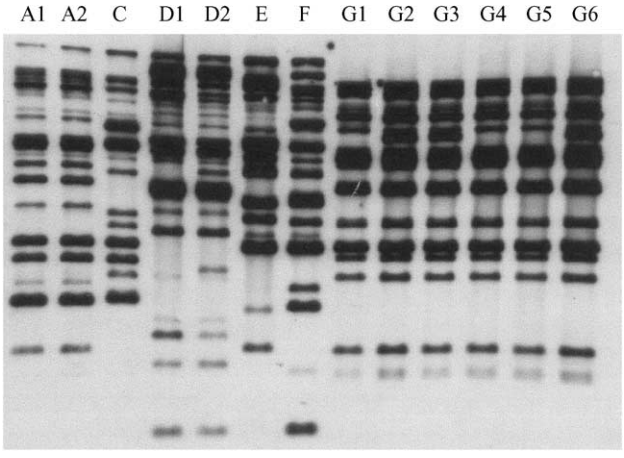

Fig. 1. DNA fingerprint pattern obtained after Southern hybridization of *Eco*RI-digested genomic DNA of *C. albicans* isolates with the *C. albicans*-specific repetitive DNA element *CARE-2* [64]. Isolates obtained from different patients (letters A–G) exhibit different fingerprints. The two isolates from patient A have an identical fingerprint, the two isolates from patient D are highly similar and represent subtypes of the same strain. Patient G harboured three subtypes of the same strain: Isolates G2, G3 and G6 are identical, isolates G4 and G5 are also identical and differ from the former by the absence of one band, and isolate G1 exhibits an additional weak band as compared to G4 and G5.

3. The molecular mechanisms of fluconazole resistance in *C. albicans*

Several different mechanisms may be responsible for the development of fluconazole resistance in *C. albicans*. These include alterations in the sterol biosynthesis pathway, overexpression of the *ERG11* gene encoding the drug target enzyme 14DM, mutations in the *ERG11* gene which result in reduced affinity of 14DM to fluconazole, and reduced intracellular drug accumulation, which is correlated with the overexpression of membrane transport proteins. In contrast, inactivation of the drug, a frequent cause of resistance to antibiotics in bacteria, has not been described as a resistance mechanism in *C. albicans*. The following sections summarize the genetic evidence that is available for the involvement of the various mechanisms in fluconazole resistance.

3.1. Alterations in the sterol biosynthesis pathway

Inhibition of 14DM by fluconazole not only results in ergosterol depletion but also in the accumulation of the methylated sterol 14α-methylergosta-8,24(28)-dien-3β,6α-diol, which inhibits cell growth [12]. Alterations in the sterol biosynthesis pathway that avoid the accumulation of this growth inhibiting sterol in the presence of fluconazole can cause fluconazole resistance. Inactivation of $\Delta^{5,6}$ desaturase (ERG3), an enzyme that acts at an earlier step than 14DM in the ergosterol biosynthesis pathway, results in altered sterole composition of the membrane (high fecosterol content) and fluconazole resistance, possibly by accumulation of 14α-methylfecosterol, which allows growth [12–14]. Recently it was shown that deletion of the *ERG3* gene in *C. albicans* resulted in reduced susceptibility of the mutants to fluconazole, providing direct genetic evidence that alteration of the sterol biosynthesis pathway can cause fluconazole resistance [15].

3.2. Mutations in the ERG11 gene encoding the drug target enzyme, 14DM

A frequent cause of drug resistance are mutations in the target structure that reduce its binding to the drug without preventing function. To identify alterations in 14DM that might cause fluconazole resistance, several investigators compared the sequence of the *ERG11* gene of fluconazole-resistant *C. albicans* strains with the published *ERG11* sequence and that of fluconazole-susceptible strains. As compared with the published *ERG11* sequence, the amino acid exchanges F105L, E266D, K287R, G448E, G450E, G464S and V488I were found only in fluconazole-resistant

isolates, but not in sensitive isolates in one study [16]. However, such sequence differences may simply reflect allelic variation and by themselves do not prove a causal relationship with resistance. Similarly, the amino acid exchanges K128T and K147R were found in sensitive isolates and were therefore suggested not to cause resistance, but such a conclusion would require a comparison of the fluconazole susceptibilities of the mutated and an otherwise identical enzyme. Alterations in the *ERG11* sequence in comparison with the published sequence in fluconazole-resistant isolates were also found by other investigators [17–19]. In these latter studies it was shown that the enzymes from the resistant isolates exhibited reduced sensitivity to inhibition by fluconazole, but the enzymes exhibited several different amino acid exchanges and it was not established which were responsible for the resistant phenotype.

Stronger evidence for mutations in the *ERG11* gene conferring fluconazole resistance was provided by comparing the sequence of the *ERG11* alleles of matched pairs of fluconazole-susceptible and resistant isolates obtained at different infection episodes from the same patient. An R467K mutation was detected in both *ERG11* alleles of a fluconazole-resistant *C. albicans* isolate as compared with matched isolates from the same patient with higher sensitivity [20]. Similarly, a G464S mutation was found in the *ERG11* alleles of an isolate displaying enhanced fluconazole resistance from another series of clinical *C. albicans* isolates [21]. Both amino acids are located near the heme binding site, and the mutations probably result in structural or functional alterations associated with the heme. The relationship of these mutations to the enhanced drug resistance of the strains was confirmed by the demonstration that higher fluconazole concentrations were needed to inhibit enzyme activity in cell free extracts of isolates containing the mutation as compared with isolates without the mutation [20,21].

A successful approach to demonstrate the involvement of *ERG11* mutations in fluconazole resistance has been the heterologous expression of different *ERG11* alleles in *S. cerevisiae* and comparing the susceptibility of the strains to fluconazole [22]. Since all the variant enzymes were expressed equally well in the heterologous host, an increased resistance should be due to differences in the amino acid sequence of the protein. The amino acid exchanges G129A, Y132H, S405F, G464S and R467K were shown to cause fluconazole resistance by this approach [22]. Using a similar strategy, an I471T exchange in *ERG11* was also shown to result in reduced fluconazole susceptibility [23], and recently an involvement in fluconazole resistance was demonstrated for additional amino acid exchanges, F126L, T229A, G307S and F449S [24]. Other mutated *ERG11* genes also conferred increased resistance on *S. cerevisiae* transformants, but these genes encoded enzymes with multiple amino acid exchanges and it could not be deduced which of them was responsible for the resistant phenotype [19,24].

Direct evidence for certain mutations resulting in decreased affinity to the drug was provided by biochemical analysis of heterologously expressed enzymes. The affinity for fluconazole of 14DM containing the mutations Y132H, G464S or R467K was reduced as compared with the wild-type enzyme, confirming that these naturally occurring mutations indeed caused drug resistance in clinical *C. albicans* isolates [25–27]. Mutations that should affect substrate or inhibitor binding can also be inferred from knowledge of the three-dimensional structure of the enzyme. Molecular modeling predicted a hydrogen bond between sterol C3-OH and the threonine at position 315 in the active center of 14DM. A T315A mutation that was introduced by design into the *C. albicans* enzyme indeed reduced its affinity to fluconazole [28]. The T315A mutation has, however, so far not been found in clinical *C. albicans* isolates.

A change from heterozygosity to homozygosity for a mutated *ERG11* gene seems to confer increased resistance. Such a change was found for clinical isolates with the G464S or the R467K mutations [20,21]. Genetic evidence suggests that a cell with two copies of R467K is significantly more resistant than a cell in which only one allele has the mutation [4]. The selection pressure exerted by the presence of fluconazole seems to favour the conversion to homozygosity once a mutation conferring enhanced fluconazole resistance has been introduced into one of the *ERG11* alleles.

Mutations in the drug target enzyme clearly are an important mechanism resulting in the emergence of fluconazole-resistant *C. albicans* strains. Table 1 provides a list of

Table 1
ERG11 mutations causing fluconazole resistance in clinical *C. albicans* strains

Mutation	Evidence for contribution to fluconazole resistance				Reference
	A	B	C	D	
F126L			+		[24]
G129A	+		+[a]		[22]
T229A	+		+		[24]
Y132H	+		+	+	[22,23,25]
G307S	+		+		[24]
S405F	+		+		[22]
F449S			+		[24]
G464S	+	+	+	+	[21,22,24,26]
R467K	+	+	+	+	[20,22,27]
I471T			+		[23]

A: Mutation found in a fluconazole-resistant isolate but not in a matched susceptible isolate from the same patient.
B: Increased fluconazole resistance of enzyme activity in cell-free extracts of isolates containing the mutation as compared with extracts from matched isolates without the mutation.
C: Expression of mutated *ERG11* gene in *S. cerevisiae* conferred higher resistance than otherwise identical gene without the mutation.
D: Biochemical analysis showed reduced affinity of the mutated 14DM for fluconazole as compared with wild-type enzyme.
[a] Conferred increased resistance only in combination with the G464S mutation, but not alone.

ERG11 mutations that have been found in clinical *C. albicans* isolates and whose contribution to fluconazole resistance has been confirmed by various experimental approaches. As stated above, more sequence variations have been found in resistant isolates, but a definite proof for their involvement in resistance remains to be established. It is also important to note that the contribution of a mutation to a resistance phenotype depends on the sequence context of the particular *ERG11* allele in which it occurs, and the effects of some mutations can be additive [22–24].

3.3. Overexpression of the ERG11 gene

In the presence of fluconazole, *C. albicans* upregulates the *ERG11* gene, presumably as a feedback mechanism to make up for ergosterol depletion [21,29]. Franz et al. [21] reported that even in the absence of fluconazole some fluconazole-resistant isolates express *ERG11* mRNA at higher levels than matched susceptible isolates in the presence of the drug. This constitutive *ERG11* overexpression seemed to contribute to fluconazole resistance, although to a lesser degree than *ERG11* mutations, since twice as much fluconazole was needed to inhibit 14DM activity in a cell free extract of such a resistant isolate to the same level as in the extract of a matched susceptible isolate [21]. *ERG11* overexpression has been found in many other fluconazole-resistant *C. albicans* isolates compared with matched susceptible isolates [24,30]. Enhanced gene expression is expected to result in higher enzyme levels and, consequently, a need for higher intracellular fluconazole concentrations to inhibit enzyme activity. There is experimental evidence that such overexpression confers fluconazole-resistance. Overexpression of *ERG11* from *C. albicans* conferred a five-fold enhanced resistance to fluconazole on *S. cerevisiae* as compared to transformants carrying the vector control [28]. When the *ERG11* gene was expressed from doxycyclin-repressible promoters in *C. glabrata*, increased levels of *ERG11* mRNA correlated with reduced fluconazole susceptibility [31]. Therefore, constitutive *ERG11* overexpression may contribute to fluconazole resistance in clinical *C. albicans* strains.

3.4. Overexpression of genes encoding efflux pumps

An important mechanism of fluconazole resistance is reduced intracellular accumulation of the drug. In recent years, it became evident that fluconazole is actively transported out of the cells in an energy-dependent manner and that an enhanced drug efflux is caused by the overexpression of genes encoding membrane transport proteins. The highly homologous genes *CDR1* and *CDR2* (*Candida* drug resistance) encode ATP-binding cassette (ABC) transporters, which use adenosine triphosphate (ATP) as the energy source, whereas the *MDR1* (multidrug resistance) and *FLU1* (fluconazole resistance) genes encode major facilitators, which use the proton gradient across the membrane as the driving force for transport. The *MDR1* gene (originally termed *BEN*^{r}) had been cloned by its ability to confer resistance to benomyl and methotrexate upon *S. cerevisiae* transformants [32], and its overexpression in *S. cerevisiae* was later shown to mediate resistance to a variety of other drugs [33]. The *CDR1* gene is a homolog of the *S. cerevisiae* pleiotropic drug resistance gene *PDR5* and was cloned by complementation of the cycloheximide hypersensitivity of an *S. cerevisiae pdr5* mutant [34]. Transformants carrying the *CDR1* gene displayed increased resistance to many other drugs, suggesting that *CDR1* also is a multidrug resistance gene. Sanglard et al. [35] demonstrated that many fluconazole-resistant, clinical *C. albicans* isolates displayed strongly increased mRNA levels of *CDR1* or *MDR1* in comparison with matched susceptible isolates and accumulated less intracellular fluconazole. They also showed that *S. cerevisiae pdr5* mutants are hypersusceptible to fluconazole and other azoles and that overexpression of *CDR1* from *C. albicans* complemented this phenotype, whereas overexpression of *MDR1* restored increased resistance to fluconazole, but not other azoles. Inactivation of *CDR1* in *C. albicans* itself resulted in enhanced intracellular fluconazole levels and increased susceptibility of the mutant to fluconazole and many other drugs, providing genetic evidence that *CDR1* is a multidrug resistance gene in *C. albicans* [36]. In contrast, disruption of *MDR1* had no effect on intracellular fluconazole accumulation and did not influence the susceptibility of the mutants to fluconazole [36,37]. However, the latter result is probably related to the fact that the strains in which *MDR1* had been inactivated, like most fluconazole-susceptible *C. albicans* isolates, did not or barely express *MDR1* under the test conditions used [11,21,29].

The *CDR2* gene, which is highly similar to *CDR1*, was identified in a screen for *C. albicans* genes that complement the hypersusceptibility of *S. cerevisiae pdr5* mutants to azoles [38]. Inactivation of *CDR2* in *C. albicans* had no effect on intracellular fluconazole accumulation and did not influence the susceptibility of the mutants to fluconazole, but this result was probably caused by the absence of *CDR2* expression in azole-susceptible *C. albicans* strains, similar to what is observed for *MDR1* (see above). However, disruption of *CDR2* in a *cdr1* mutant background further increased the susceptibility to fluconazole and other drugs. Interestingly, derivatives of a *cdr1* mutant that regained wild-type levels of fluconazole-susceptibility expressed the *CDR2* gene, and *CDR2* overexpression was also observed in fluconazole-resistant clinical *C. albicans* isolates [38]. Therefore, *CDR2* encodes a multidrug resistance gene that can mediate resistance to fluconazole and other drugs in *C. albicans*.

The *CDR* gene family in *C. albicans* comprises many more genes, but apart from *CDR1* and *CDR2* no evidence for the involvement of other members of the gene family in fluconazole resistance has been obtained so far [39,40]. Recently, a gene that is homologous to *MDR1*, *FLU1*, has

been isolated by its ability to confer fluconazole resistance on hypersusceptible *S. cerevisiae* transformants [41]. Inactivation of *FLU1* in a *C. albicans* strain in which several other multidrug resistance genes had been deleted resulted in increased susceptibility to fluconazole, demonstrating that *FLU1* can mediate fluconazole resistance in *C. albicans*. However, overexpression of *FLU1* has not yet been found as a cause of fluconazole resistance in clinical *C. albicans* isolates.

Overexpression of efflux pumps encoded by the *CDR1*, *CDR2* and *MDR1* genes has been shown to be one of the most frequent mechanisms of fluconazole resistance [8,11,21,24,30,35,38,42,43], but the exact mechanism how resistance is mediated has not been resolved, nor is the physiological function of these membrane transport proteins known. Recent studies suggested that Cdr1p transports phospholipids from the inner to the outer leaflet of the cytoplasmic membrane, thereby changing the membrane structure [44]. Earlier studies had demonstrated that reduced intracellular fluconazole accumulation was associated with an altered membrane structure, presumably resulting in reduced drug uptake [45]. The resistance phenotype associated with overexpression of membrane transport proteins may therefore not only be caused by enhanced efflux, but could in part also be due to membrane alterations resulting in reduced drug uptake, a possibility that has not been investigated so far and should be kept in mind.

3.5. Multiple mechanisms contribute to a stepwise development of fluconazole resistance in C. albicans

Each of the mechanisms described above can cause reduced susceptibility of *C. albicans* to fluconazole. Several studies have shown that multiple mechanisms may be combined to result in a stepwise development of fluconazole resistance, which ultimately becomes clinically relevant and causes therapy failure. For example, White [30] described gradual increases in fluconazole resistance in serial isolates of a *C. albicans* strain by overexpression of *MDR1*, *ERG11*, and one or more of the *CDR* genes, combined with the aquisition of the R467K mutation in *ERG11*. Sanglard et al. [22] found different combinations of *ERG11* mutations with overexpression of *MDR1*, *CDR1* or *CDR2*, in other series of *C. albicans* isolates, and Franz et al. [21] reported two series of clinical *C. albicans* isolates in which *MDR1* overexpression was combined with either the G464S mutation or *ERG11* overexpression. Combinations of different resistance mechanisms have recently been reported to be responsible for fluconazole resistance in the majority of clinical *C. albicans* strains studied [24]. It is therefore evident that the generation of a highly resistant strain from a highly susceptible strain is the result of multiple mechanisms, each of which contributes only partially to the resistant phenotype. Whether a particular alteration creates a clinically resistant *C. albicans* strain therefore depends on the genetic background in which this alteration occured, i.e. if the strain already exhibited other mutations resulting in decreased susceptibility, even when the previous changes did not cause a clinically relevant resistance level. For example, some *ERG11* mutations which have been shown experimentally to cause fluconazole resistance have also been found in isolates with MICs in the susceptible range [24], and the overexpression of genes encoding efflux pumps is not necessarily sufficient to result in clinically relevant resistance.

4. The regulation and role of the *MDR1* gene in *C. albicans* fluconazole resistance

In the presence of fluconazole, *C. albicans* induces expression of the *ERG11* gene above its normal level, presumably in response to ergosterol limitation [21,29]. In contrast, the genes encoding efflux pumps, *MDR1*, *CDR1* and *CDR2*, are not activated under same conditions [21,29]. However, in many fluconazole-resistant clinical *C. albicans* isolates, these genes are constitutively overexpressed, indicating that mutations must have occurred in these strains that abolish the normal regulation of the genes. Such mutations could either occur in the promoter region of the genes themselves or involve trans-regulatory factors. The comparison of the promoter sequences of both *MDR1* alleles of two matched pairs of clinical *C. albicans* isolates in which fluconazole resistance correlated with constitutitve *MDR1* expression did not reveal promoter mutations that might be responsible for *MDR1* activation in the resistant isolates [46]. Direct evidence for the involvement of an altered regulatory factor was obtained by integrating a green fluorescent protein (*GFP*) reporter gene under the control of

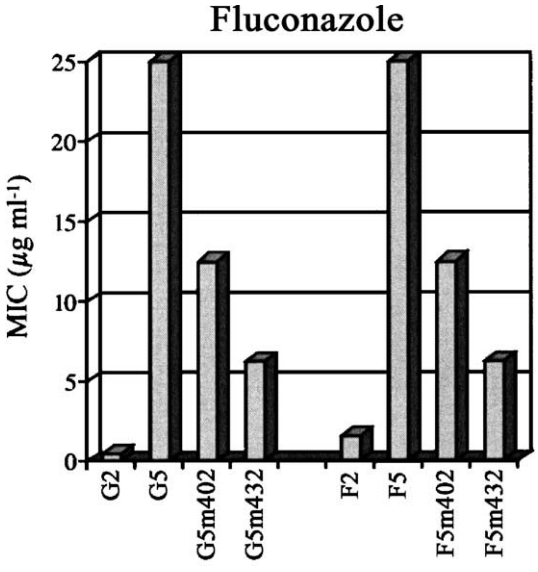

Fig. 2. Minimal inhibitory concentration of fluconazole for the fluconazole-susceptible *C. albicans* isolates F2 and G2, the matched resistant isolates F5 and G5, and the heterozygous (F5M402 and G5M402) and homozygous (F5M432 and G5M432) *mdr1* mutants derived from the resistant isolates.

the *MDR1* promoter from a fluconazole-susceptible *C. albicans* strain at an ectopic site into the genome of the two matched pairs of clinical isolates. Although the *MDR1* promoter controlling *GFP* expression was identical in all four reporter strains, only the fluconazole-resistant isolates, but not the matched susceptible isolates, expressed the GFP [46]. Therefore, the activation of the *MDR1* gene in these fluconazole-resistant isolates must have been caused by mutations in regulatory factors, and this is likely to be true also for other fluconazole-resistant *C. albicans* isolates and other genes encoding efflux pumps. Several *C. albicans* genes encoding regulatory factors that influence the expression of efflux pumps have been identified, for example the *CAP1* gene, which is homologous to *YAP1* from *S. cerevisiae*, or the *FCR1*, *FCR2* and *FCR3* genes [47–49]. However, there is no evidence so far for these transcriptional regulators being the cause of the constitutive overexpression of efflux pumps in clinical *C. albicans* strains.

The involvement of regulatory factors in *MDR1* overexpression pointed to the possibility of a simultaneous activation of additional genes, including genes encoding yet unknown efflux pumps. Therefore, the activation of other genes than *MDR1* might be the cause of fluconazole resistance. In fact, *MDR1* disruption in *C. albicans* laboratory strains did not enhance the susceptibility of the mutants to fluconazole [36,37]. However, these strains did not detectably express the *MDR1* in vitro and, as noted above, fluconazole also does not induce *MDR1* expression in *C. albicans*. To assess the contribution of *MDR1* overexpression to fluconazole resistance, it was therefore necessary to inactivate the gene in fluconazole-resistant, clinical *C. albicans* isolates. Until recently, the gene disruption

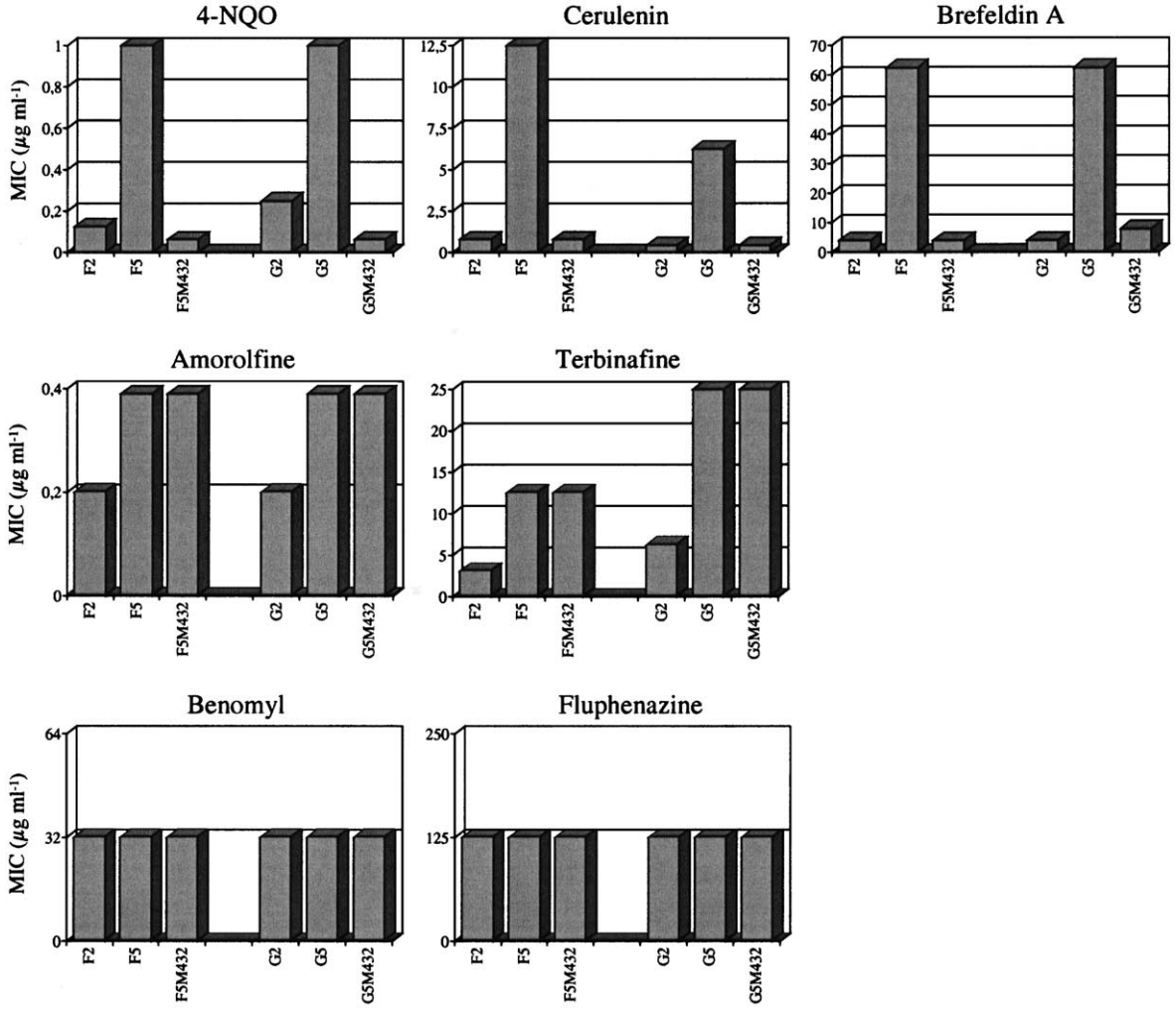

Fig. 3. Susceptibility of the matched fluconazole-susceptible and resistant clinical *C. albicans* isolate pairs (F2/F5 and G2/G5) and the *mdr1* null mutants F5M432 and G5M432 derived from the resistant isolates F5 and G5, respectively, to other drugs. The enhanced resistance of isolates F5 and G5, as compared with F2 and G2, to 4-NQO, cerulenin, and brefeldin A is due to *MDR1* overexpression, because it is abolished by deletion of the *MDR1* gene. The slightly increased resistance of F5 and G5 to amorolfine and terbinafine was not affected by *MDR1* deletion, and therefore, must have been caused by other genomic alterations than *MDR1* overexpression. The susceptibilities of the strains to fluphenazine and benomyl remained unchanged by either *MDR1* overexpression or deletion of the gene in the *MDR1* overexpressing isolates.

approach in *C. albicans* was confined to auxotrophic *ura3* mutants, but could not conveniently be performed in wild-type strains [50]. The development of a mutagenesis procedure that is based on the use of a recyclable, dominant selection marker allowed targeted gene inactivation also in clinical *C. albicans* isolates [51]. Disruption of the *MDR1* gene in two different, *MDR1* overexpressing *C. albicans* isolates resulted in enhanced susceptibility of the mutants to fluconazole, providing direct genetic evidence that *MDR1* overexpression contributes to fluconazole resistance in clinical *C. albicans* isolates [51] (Fig. 2).

5. *MDR1* overexpression mediates multidrug resistance in *C. albicans*

The results of experiments in which *MDR1* overexpression in hypersusceptible *S. cerevisiae* strains conferred resistance to a variety of structurally unrelated drugs in addition to fluconazole suggested that Mdr1p transports many different toxic substances. Consequently, deletion of the *MDR1* gene in a *C. albicans* strain that expressed high *MDR1* mRNA levels resulted in an enhanced susceptibility of the mutant to several of these drugs [52]. Interestingly, however, *mdr1* deletion in this strain had no effect on the susceptibility to benomyl, a drug that was thought to be an Mdr1p substrate from the heterologous expression studies. This result was interpreted as *C. albicans* having other resistance mechanisms, presumably efflux systems that compensate for the loss of the *MDR1* gene. Clinical *C. albicans* isolates which overexpressed *MDR1* were also more resistant to other drugs in addition to fluconazole, e.g. 4-nitroquinoline-*N*-oxide (4-NQO), cerulenin, and brefeldin A, as compared with matched isolates that did not detectably express *MDR1* in vitro [53] (Fig. 3). The increased resistance was abolished when the *MDR1* gene was deleted from the genome of these isolates, providing genetic evidence that *MDR1* overexpression in clinical *C. albicans* isolates indeed confers resistance to various, structurally unrelated drugs [53]. However, *MDR1* overexpression in such clinical isolates did not enhance resistance to some other drugs that were thought to be Mdr1p substrates, e.g. amorolfine, terbinafine, fluphenazine and benomyl, and *MDR1* deletion did not influence the susceptibility of the strains to these drugs (Fig. 3). Even if *C. albicans* possesses other mechanisms of resistance to these toxic compounds one would expect that, if Mdr1p can transport these substances out of the cell, a strong overexpression of *MDR1* should further increase resistance, similar to its contribution to fluconazole resistance, which is often mediated by several additive mechanisms. These findings, together with the absence of an effect of *MDR1* deletion, suggests that heterologous expression studies may in some cases give misleading results about the substrate spectrum of the efflux pumps in *C. albicans*. The expression of *C. albicans* membrane proteins in *S. cerevisiae* host strains may alter the structure of the cell membrane, especially in strains in which one or several of their own genes encoding membrane proteins were deleted, and these alterations might indirectly influence the susceptibility of the cells to toxic compounds. As mentioned above, such alterations in membrane structure have been observed after overexpression of the *CDR1* gene in *S. cerevisiae* [44]. It should also be noted that no direct proof has yet been provided that the *C. albicans* efflux pumps bind and transport antifungal drugs. Whatever the mechanism is, there is now convincing evidence that the overexpression of *MDR1*, and also the *CDR1* and *CDR2* genes, contributes to the fluconazole-resistant phenotype of clinical *C. albicans* isolates.

6. Conclusions and perspectives

Considerable efforts have been undertaken in the past years to unravel the molecular mechanisms of fluconazole resistance in *C. albicans*, and this knowledge should help to devise strategies to overcome the resistance problem. Mutations resulting in reduced affinity of the target enzyme 14DM to fluconazole have been uncovered, but the precise way how the amino acid exchanges influence the binding of the drug can only be fully understood when more structural information on wild-type and mutated enzymes becomes available. This information may allow the design of drug derivatives whose binding is not affected by the resistance mutations.

The development of efflux pump inhibitors that could be used in combination with fluconazole is a promising strategy to abolish resistance of strains overexpressing these transporters [54]. Interestingly, *S. cerevisiae* transformants expressing *CDR1* from *C. albicans* exhibited increased sensitivity to peptidic antifungals and other drugs [34,55]. Although similar findings have not been reported for *CDR1* overexpressing *C. albicans*, these observations suggest that the development of drug resistance may also create new vulnerable sites which may be exploited to preferentially attack fluconazole-resistant strains. The mutations responsible for overexpression of the genes encoding efflux pumps in fluconazole-resistant clinical *C. albicans* isolates are still unknown, but presumably involve alterations in regulatory proteins [46]. The elucidation of the regulatory pathways controlling expression of these genes may provide a rationale basis for approaches to interfere with their activation and thus overcome resistance.

Mutations resulting in drug resistance provide a selective advantage in the presence of the drug. However, some of the described alterations might reduce the fitness of the resistant strains under non-selective conditions, for example *ERG11* mutations that also lower 14DM activity [26,27] or the unregulated expression of genes encoding efflux pumps, which is tightly controlled in the parental strains [46]. Some fluconazole-resistant isolates in fact exhibit reduced virulence, but virulence may also remain unaltered or even be

increased [56,57]. Fluconazole-resistant strains can loose the resistant phenotype during propagation in drug-free medium, arguing for a selective disadvantage of some resistance mutations [42]. On the other hand, it was recently also demonstrated that the costs of resistance can be overcome during further evolution of in vitro generated fluconazole-resistant *C. albicans* strains [58]. It will be interesting to test whether clinical *C. albicans* having different resistance mutations may display a reduced fitness as compared with their drug-sensitive parents within the complex environment of the host. Adequate treatment strategies might reduce the frequency of resistant strains [59]. An important research area also is the intrinsic resistance of *C. albicans* to fluconazole when the organism grows as a biofilm [60–62]. The basis of biofilm resistance, which seems to be independent of the expression of efflux pumps [63], and its implications for the treatment of *Candida* infections remain to be elucidated.

Acknowledgements

The author's work is supported by grants from the Deutsche Forschungsgemeinschaft (DFG grants MO846/2 and MO846/3).

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